Skin Contact Irritation Conditions the Development and Severity of Allergic Contact Dermatitis

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Irritant contact dermatitis (ICD) is a frequent inflammatory skin disease induced by skin contact with low molecular weight chemicals such as haptens endowed with proinflammatory properties. Allergic contact dermatitis (ACD) is a frequent complication of ICD and is mediated by hapten-specific T cells primed in lymph nodes by skin emigrating dendritic cells. The aim of this study was to analyze the relationship between ICD and ACD to 2,4-dinitrofluorobenezene (DNFB) in C57BL/6 and BALB/C mice, which develop a severe and a moderate skin inflammation, respectively. Upon a single skin painting with DNFB, C57BL/6 developed within hours a more severe dose-dependent ICD response as compared to BALB/C mice, which was associated with enhanced upregulation of IL-1β, IL-6, and IL-10. Skin exposure to a low dose of DNFB resulted, in both strains, in a low ICD that resolved in a few hours. Alternatively, skin painting with either an intermediate or a high DNFB concentration induced an ICD that subsequently gave rise to an ACD reaction whose intensity was proportional to the magnitude of the ICD response and was more severe in C57BL/6 mice than in BALB/C mice. In conclusion, the hapten-induced skin contact irritation conditions the development and the severity of ACD.

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INTRODUCTION

Contact dermatitis (CD) is a frequent inflammatory skin disease induced by skin exposure to low molecular weight chemicals, endowed with both proinflammatory and antigenic properties (Rowland *et al.*, 2001; Saint-Mezard *et al.*, 2004b). Through their proinflammatory properties, haptens generate a non-antigen-specific skin inflammation also known as irritant contact dermatitis (ICD) (Basketter *et al.*, 1999). Following contact with the skin, haptens cause activation of skin cells resulting in the rapid production of a whole array of inflammatory cytokines (including IL-1β, IL-6, and TNF-α) and chemokines (including CCL20) involved the recruitment and activation of dendritic cell (DC) precursors and in the migration of skin DC to draining lymph nodes (LNs) (for review see Pastore *et al.*, 2004; Saint-Mezard *et al.*, 2004b). In addition, haptens become immunogenic after covalent binding to discrete amino-acid residues of proteins and by generating modified self-proteins to induce allergic contact dermatitis (ACD) (Lepoittevin and Leblond, 1997; Smith Pease *et al.*, 2003). ACD, also referred to as contact sensitivity is a delayed-type hypersensitivity reaction mediated by hapten-specific T cells, which are primed in LNs and recruited in the skin during the afferent and efferent phase of the reaction, respectively (Blauvelt *et al.*, 2003; Saint-Mezard *et al.*, 2004b). Upon skin contact, the hapten is taken up by immature skin DCs which migrate from the skin to the paracortical area of draining LNs, where they prime hapten-specific T cells through presentation of hapten–protein complexes on major histocompatibility complex molecules (Bour *et al.*, 1995; Krasteva *et al.*, 1998). A subsequent skin challenge with the same hapten leads to the rapid recruitment of effector T cells in the skin, that initiate inflammation via induction of keratinocyte apoptosis (Bour *et al.*, 1995; Kehren *et al.*, 1999). We and others have demonstrated that ACD to the strong hapten 2,4-dinitrofluorobenzene (DNFB) in mice is mediated by CD8+ cytotoxic T cells and downregulated by CD4+ T cells (Gorbachev and Fairchild, 2001; Girolomoni *et al.*, 2004; Saint-Mezard *et al.*, 2004a).

Clinical evidence supports the hypothesis that ICD promotes the development of ACD and conditions its severity (Uter *et al.*, 2005). Furthermore, it is widely accepted that the susceptibility of individuals to develop ACD directly correlates with the proinflammatory properties of haptens (Smith *et al.*, 2002). The severity of ACD can greatly vary from a mild and transient skin inflammation with pruritus and redness only to a severe long lasting exudating dermatitis. Factors which control the severity of ACD include the nature of haptens (ranging from strong to weak sensitizers), their

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Abbreviations: ACD, allergic contact dermatitis; B6, C57BL/6; BALB, BALB/C; DC, dendritic cell; DNFB, 2,4-dinitrofluorobenezene; ICD, irritant contact dermatitis; LN, lymph node; mRNA, messenger RNA; TNCB, 2,4,6-trinitrochlorobenzene

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concentrations and the conditions of exposure, most of ACD responses being observed in occupational settings (Kimber *et al.*, 2003). Indeed, haptens comprise very diverse chemicals, including a limited number of strong contact sensitizers able to sensitize more than 90% of naive individuals after a single skin contact and thousands of weak haptens that can induce sensitization in a small proportion of individuals only (occupational disease) (Diepgen and Kanerva, 2006; Pierard-Franchimont *et al.*, 2006). Although the ability of a given hapten to induce ACD is linked to its antigenicity, its intrinsic proinflammatory property is essential for efficient priming of specific T-cell precursors (Enk and Katz, 1992; Flint *et al.*, 1998; Le Borgne *et al.*, 2006; personal data). It has been known for nearly 40 years that irritation/inflammation can enhance ACD responses. Experimental studies in guinea-pig models (Magnusson and Kligman, 1969) as well as in human maximization tests (Kligman, 1966) have shown that co-administration of adjuvants or irritants with haptens could markedly increase the hapten-induced ACD reaction. However, evidence that the quality and magnitude of the ACD reaction depends on the nature and strength of the inflammatory signals delivered by haptens during the course of ICD reactions is still lacking.

Here, we investigated the relationship between ICD and ACD reactions in C57BL/6 (B6) and BALB/C (BALB) mice, which develop a severe or mild skin inflammation to DNFB, respectively. We used the model of primary contact hypersensitivity induced by a single DNFB painting on the ear (Saint-Mezard *et al.*, 2003). In this model, persistence of the hapten in the skin for several days leads within 6 days to the development of hapten-specific T cells which can infiltrate the sensitized skin site and induce a contact hypersensitivity reaction. Therefore, in primary contact hypersensitivity, the magnitude of the skin inflammation at day 6 depends solely on the antigenic and proinflammatory signals delivered by hapten exposure at day 1, allowing a direct comparison of both ICD and ACD responses in different strains of mice. We show that ICD responses are dose-dependent and much higher in B6 than in BALB mice. More importantly, the intensity of the ACD reaction is correlated to that of the ICD response, demonstrating that the hapten-induced skin contact irritation conditions the development and the severity of ACD.

RESULTS

ICD responses are more severe in C57BL/6 mice than in BALB/C mice

Different doses of DNFB (0.1, 0.3, or 0.5%) applied onto the ear induced an ICD reaction manifested by ear swelling, which developed within 1 hour after DNFB painting in both BALB and B6 mice. The ICD reaction was dose dependent and presented as a mild, moderate and severe skin inflammation in response to low, intermediate and high DNFB concentrations, respectively (Figure 1a and b). The ICD reactions were dramatically higher in B6 (Figure 1b) than in BALB mice (Figure 1a). Indeed, at each DNFB concentration, ear swelling was 3-fold higher in B6 skin as compared to BALB mice. Thus, the skin of B6 mice seems to be more sensitive to the proinflammatory effects of DNFB than that of BALB mice.

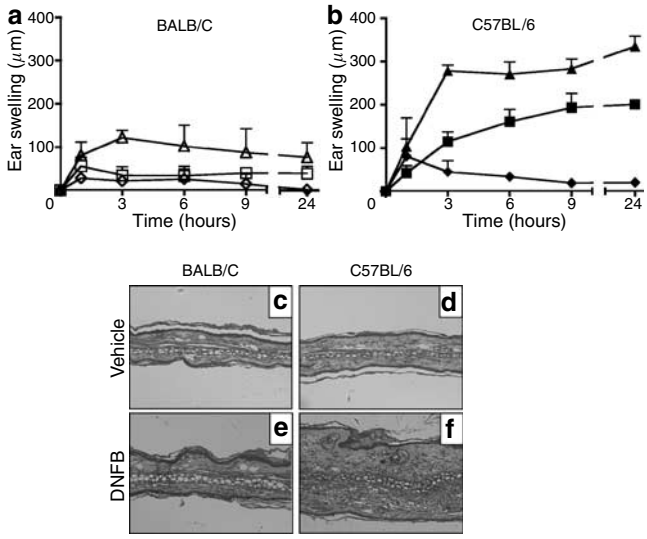

Figure 1. Skin contact irritation is more severe in C57BL/6 mice than in BALB/C mice. A single skin painting with 0.1% (◇), 0.3% (□) or 0.5% (△) DNFB on the left ear (and vehicle on the right ear) of (**a**) BALB/C (white) and (**b**) C57BL/6 (black) mice induced within hours an immediate ear skin inflammation. Results are expressed as the mean ear swelling ± SD. (**c–f**) Histological analysis of ear sections from BALB/C and C57BL/6 mice, 6 hours after vehicle (**c, d**) or DNFB (**e, f**) sensitization. Hematoxylin and eosin staining. Original magnification ×100. Results are representative of three independent experiments.

The peak and the kinetics of the ICD reaction varied between the two strains. In BALB mice, the ICD peaked between 1 and 3 hours irrespective of the dose and decreased thereafter. Alternatively, in B6 mice, only low DNFB concentration induced an ICD reaction with a peak at 1 hour; intermediate and high concentrations of DNFB resulted in an ICD peak delayed at 3–6 hours with persistence of the skin inflammation up to 24 hours after sensitization.

Histological analysis of 0.3% DNFB-treated ears at 6 hours after skin painting confirmed that the skin inflammation, as revealed by dermal edema and cell infiltration in the dermis, was more pronounced in B6 (Figure 1f) than in BALB mice (Figure 1e).

Collectively these results show that B6 mice have a ''high ICD responder'' status whereas BALB mice have a ''low ICD responder'' status.

The severity of ICD correlates with high levels of IL-1β/IL-6 mRNA and low levels of IL-10 mRNA

To get better insights into the molecular mechanisms involved in the development of ICD reactions we next analyzed, by RNA protection assay, the expression of proinflammatory (IL-1α, IL-1β, and IL-6) and anti-inflammatory (IL-10) cytokines in the ear skin, 3 hours after skin exposure to DNFB. The ear skin of naive and vehicle-treated BALB mice contained only trace amounts of IL-1α, IL-1β, IL-6, and IL-10 messenger RNA (mRNA) (Figure 2). DNFB sensitization induced a dose-dependent upregulation of both

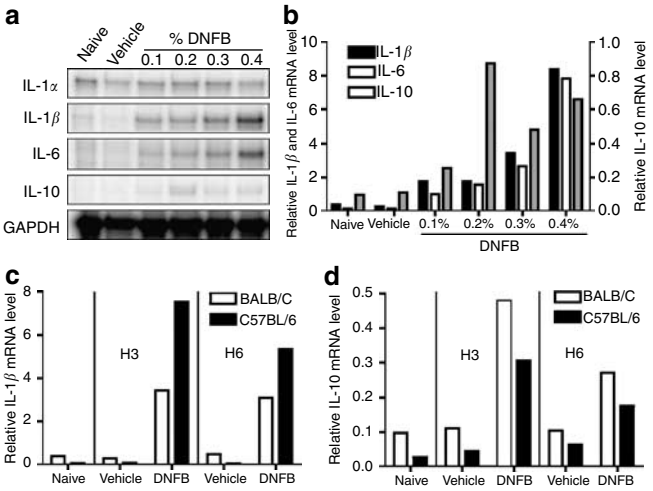

Figure 2. The severity of ICD correlates with high levels of IL-1β/IL-6 mRNA and low levels of IL-10 mRNA. (**a**) Detection and quantification of mRNA for IL-1α, IL-1β, IL-6, and IL-10 by RNAse protection assay in the skin of DNFB-treated BALB/C mice 3 hours after sensitization. Controls include expression of mRNA in the ear skin of naive mice and of vehicle-treated mice at 3 hours. (**b**) Histograms representations of the relative quantities of IL-1β (black bars), IL-6 (white bars), and IL-10 (gray bars) mRNA recovered from the different experimental conditions, as compared to expression of glyceraldehyde-3-phosphate dehydrogenase mRNA as reference gene. (**c**, **d**) Comparison of the expression levels of (**c**) IL-1β and (**d**) IL-10 mRNA levels by RNase protection assay in the skin of BALB/C (white bars) and C57BL/6 (black bars) mice, 3 and 6 hours following 0.3% DNFB sensitization. Controls include mRNA levels in the ear skin of naive mice and of vehicle-sensitized mice. mRNA relative quantities were compared with glyceraldehyde-3-phosphate dehydrogenase mRNA as standards.

IL-1β and IL-6 (Figure 2b) with a 2- and 4-fold increase in the levels of both cytokines induced by 0.3 and 0.4% DNFB, respectively, as compared to 0.1% DNFB. IL-10 mRNA upregulation was lower than that of IL-1β and IL-6. No significant upregulation of IL-1α mRNA was observed at any DNFB concentration (Figure 2a). Similar upregulation of IL-1β and IL-6, and too a lesser extend of IL-10, was found in DNFB-painted B6 mice (data not shown). These results show that in both mouse strains, the intensity of ICD is correlated with the production of IL-1β and IL-6 by skin cells.

Next, we compared the levels of IL-1β and IL-10 in the skin of B6 and BALB mice at 3 and 6 hours after DNFB painting. The high ICD responder B6 mice produce twice more IL-1β mRNA than BALB mice (Figure 2c). Alternatively, expression of IL-10 mRNA was consistently higher in the low ICD responder BALB as compared to B6 mice (Figure 2d). These results suggest that the differential production of pro- and anti-inflammatory cytokines may explain the differences in the intensity of ICD reactions observed in B6 and BALB mice.

The intensity of ICD reaction dictates the severity of ACD responses

Mice were sensitized by DNFB and ear swelling was measured the following hours (for ICD) and every day from day 4 until day 16 (for ACD). As previously observed, B6

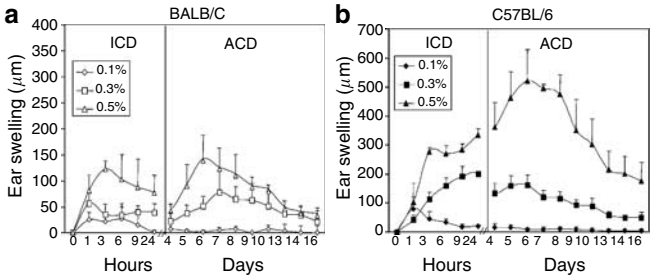

Figure 3. The intensity of ICD reaction dictates the severity of ACD responses. DNFB-induced skin inflammation was analyzed in groups of five (**a**) BALB/C and (**b**) C57BL/6 mice within hours (left panel) and days (right panel) following sensitization with DNFB 0.1% (◇, ◆), 0.3% (□, ■), or 0.5% (△, ▲) on the left ear and vehicle on the controlateral ear. Results are expressed as the mean ear swelling ± SD. Data are representative of four experiments.

mice developed a more intense dose-dependent ICD as compared to BALB mice (Figure 3a and b, left panel). In both strains, intermediate and high DNFB concentrations induced typical ACD responses characterized by bell-shaped ear swelling curves, which peaked between day 6 and 8 depending on the hapten concentration and on the mouse strain (Figure 3a and b, right panel). Downregulation of ACD reaction started at day 9 and was almost complete by day 16. For each mouse strain, the intensity of ACD reactions was proportional to the concentration of DNFB used for sensitization and therefore directly correlated to the magnitude of the ICD reaction. Consequently, B6 mice developed more severe ACD reactions than BALB mice. Of note, a low DNFB concentration was responsible for an early ICD reaction but was unable to induce an ACD reaction irrespective of the mouse.

As we have previously shown that the ACD is mediated by IFN-γ-producing CD8+ T cells primed in draining LNs and recruited in the skin (Kehren *et al.*, 1999), we next analyzed the T-cell response in the skin and auricular draining LNs of B6 and BALB mice sensitized with DNFB. We observed a direct correlation between (i) the severity of the ICD reaction, (ii) the numbers of DNFB-specific CD8+ T cells in LNs (enzyme-linked immunosorbent spot assay), (iii) the magnitude of the recruitment of IFN-γ-producing CD8+ T cells (RT-PCR analyses), and (iv) the intensity of ACD at day 6 (data not shown).

DISCUSSION

This study shows that the development and severity of ACD depend on the quality and magnitude of the ICD reaction. Skin exposure to increasing doses of DNFB induced a dose-dependent ICD, which developed as early as 1 hour after skin painting and resolved in a few hours to a few days depending on the DNFB concentration. More importantly, the intensity of ACD was proportional to the concentration of DNFB used for sensitization and therefore directly correlated to the magnitude of the ICD reactions. B6 mice were more sensitive than BALB mice to the proinflammatory effect of DNFB and exhibited a 3-fold increase in both immediate ICD and late-ACD responses.

ICD is the clinical outcome of the rapid release of proinflammatory cytokines in skin sites exposed to haptens that promote local recruitment of inflammatory cells from blood. *In vivo* and *in vitro* studies have documented that haptens can activate skin cells and induce the production of several cytokines including IL-1β which, by stimulating the migration of skin DC to LNs, has a key role in sensitization. Lack of IL-1β results in failure to initiate ACD (Enk *et al.*, 1993; Shornick *et al.*, 1996; Effendy *et al.*, 2000). Alternatively, IL-10 is an important down-regulatory factor for ACD and acts by inhibiting effector T-cell activation. Our data confirm previous studies showing the rapid upregulation of IL-1β, IL-6, and IL-10 by skin cells exposed to strong haptens (Enk and Katz, 1992; Kondo *et al.*, 1994) and further show that the intensity of ICD is proportional to the level of IL-1β and IL-6 transcripts, both in B6 and BALB mice. More importantly, B6 and BALB mice, which develop severe and mild ICD reactions, respectively, express an opposite pattern of production of IL-1β and IL-10. The IL-1β/IL-10 ratio is dramatically higher in B6 than in BALB mice, suggesting that the strain differences in the intensity of ICD could rely on the intrinsic ability of a mouse strain to produce proinflammatory rather anti-inflammatory cytokines following hapten stimulation.

Besides cytokine production, hapten sensitization leads within a few hours to the recruitment from blood of myeloid DC precursors and migration of immature skin DC to LNs. We and others have recently shown that hapten-induced recruitment of blood DC precursors into the skin dictates the severity of ACD (Le Borgne *et al.*, 2006; personal data). Interestingly, we found that upon DNFB painting, more blood-derived DC precursors are recruited from blood into skin in the high ACD responder B6 mice as compared to the low ACD responder BALB mice. Consequently, higher numbers of skin emigrating DC reaching the draining LN and enhanced numbers of effector T cells were primed in B6 mice versus BALB mice. Thus, the correlation between the irritant properties of haptens and the magnitude of the ACD reaction suggests that the skin recruitment of DC precursors results from the adjuvant properties of haptens.

The initial dogma according to which immunity results from ''self non-self-discrimination'' has recently been challenged by the ''danger signal'' model, which proposes that the strength of inflammatory signal delivered by the immunogen dictates the outcome of immunization on sensitization or tolerance (Matzinger, 2002). Haptens, by modifying self-proteins generate new antigenic motifs presented to specific T-cell precursors. However, not all haptens are capable of sensitization after a single skin exposure and we have reported that weak haptens such as fragrance allergens are unable to prime ACD effector T cells (Vocanson *et al.*, 2006). Indeed, weak haptens can lead to sensitization under specific conditions only, including higher and more frequent exposures. Thus, recognition of hapten-modified self-peptides by T cells does not preclude induction of hapten-specific T-cell-mediated skin inflammation. Alternatively, our data support that sufficient ''danger signal'' provided by haptens at the site of sensitization and manifested by proinflammatory cytokine and chemokine production is a prerequisite for generation of ACD. That strong haptens are able to deliver potent danger signals to skin innate immunity was recently demonstrated by Le Borgne *et al.* (2006) who showed that DNFB behaves as an adjuvant able to promote the priming of CD8+ T cells specific to an irrelevant co-administered protein antigen. Along these lines, Grabbe *et al.* (1996) reported that a given hapten could promote induction of an ACD reaction in response to a non-cross-reactive hapten. Indeed, oxazolone-specific ACD could develop in oxazolone-sensitized mice upon challenge with both oxazolone and (2,4,6-trinitrochlorobenzene TNCB) at infra-optimal doses unable to sensitize for ACD. Our data further emphasize that the danger signal provided by the strong hapten DNFB is dose dependent and conditions the intensity of the ICD reaction. Indeed, the two high DNFB concentrations (0.3 and 0.5%) induced dose-dependent ICD leading to ACD responses, whereas the low DNFB dose (0.1%) resulted in a mild ICD reaction, correlated with low levels of IL-1β, unable to give rise to an ACD response. As previously shown, decreasing further the DNFB concentration to as low as 0.01%, a concentration devoided of irritant properties, is responsible for tolerance to DNFB, which prevents the development of ACD to high DNFB concentrations (Maurer *et al.*, 2003). Collectively, these observations strongly support that the adjuvant properties of strong haptens contribute in both ICD and ACD and further emphasize that ''danger'' signals depending on the intrinsic proinflammatory properties of the hapten and on the skin sensitivity of the host are required for induction of the initial ICD reaction that contributes to the severity of ACD.

Although the reason why different individuals develop severe or mild ACD reactions in response to identical hapten stimulation is not known, our results indicate that the genetic susceptibility to the adjuvant effect of haptens is the crucial parameter that conditions the magnitude of ACD. B6 skin cells appears more sensitive to the DNFB-induced inflammatory signals than BALB skin cells inasmuch as they produce higher levels of IL-1β and IL-6 (Figure 2) and increased amounts of CCL20, a chemokine involved in the recruitment of blood DC precursors into the skin (Le Borgne *et al.*, 2006; personal data). In addition, the variable susceptibility to ACD in B6 and BALB mice could be explained by strain-dependent differences in mast cell and macrophage functions. Indeed, following stimulation with Toll-like receptor ligands like lipopolysaccharide, peritoneal macrophages of B6 mice produce higher levels of TNF-α (Watanabe *et al.*, 2004), which induces DC migration to LNs. In addition, mast cells were shown to contribute to ACD via release of TNF-α and macrophage inflammatory protein-2 (Biedermann *et al.*, 2000). At the basal state, mast cells are more abundant in skin of B6 than of BALB mice and produce, following UV stimulation (Hart *et al.*, 1998) and anti-IgE induced activation (Noguchi *et al.*, 2005), increased levels of immediate mediators including histamine and β-hexosaminidase. As histamine is involved in vasodilatation and vasopermeation, its enhanced secretion by B6 mast cells

may also contribute to the increased dermal edema by allowing more efficient recruitment of blood cells, through interaction with H1-receptors on endothelial cells.

In conclusion, our data demonstrate that the development and severity of ACD depend on early inflammatory events occurring within hours after sensitization and suggest that genetic susceptibility of ACD in human may result from differences in host sensitivity to the adjuvant effect of haptens that could explain the diversity of clinical presentations of ACD among patients.

MATERIALS AND METHODS

Mice

Female C57BL/6 and BALB/c mice (7–10 weeks of age) were purchased from Charles River Laboratories (L'Arbresle, France). Animals were left to acclimate for 1 week before entering the study. Five mice were used per group. Mice were provided food and water *ad libidum*. All experimental procedures were in accordance with the CREEA (Comité régional d'éthique pour l'expérimentation animale) guidelines on animal welfare.

Reagents

DNFB (Sigma, Saint Quentin Fallavier, France) was diluted in acetone:olive oil vehicle (4:1 vol/vol), freshly before application.

Assay for primary allergic contact sensitivity

The model has been described extensively elsewhere (Saint-Mezard *et al.*, 2003). Naive mice were sensitized at day 0 by a single application of various doses of DNFB applied on the left ear, whereas the same volume of vehicle was applied on the right ear. At various time after ear sensitization, ear thickness was measured with a spring-loaded micrometer (J15, Blet SA, Lyon, France). Ear swelling was calculated by subtracting the initial value from the value recorded on the corresponding time, and further subtracting any swelling recorded for vehicle-control ear from ear swelling recorded for the hapten-applied ear.

Histology

Ears were fixed in a 3% formalin solution for 24 hours and processed whole through a routine 15 hours cycle to paraffin wax embedding. Sections (4 μm) were cut using a microtome and mounted on Superfrost® Plus slides. Sections were dried overnight at 37°C. The slides were dewaxed in Ottix® baths and staining according to routine hematoxylin and eosin staining procedure.

mRNA extraction and multiprobe RNAse protection assays

At 3 and 6 hours after the ear painting, ear samples were collected from vehicle and DNFB-treated mice and frozen in liquid nitrogen. Total RNA was extracted using the RNAXEL kit (Eurobio, F-91953, Les Ulis, France). Chemokines mRNA levels were measured by RNase protection assays using the Riboquant kit (BD Pharmingen, San Diego, CA) following the instructions of the supplier. The quantity of protected mRNA was determined using a PhosphorImager and ImageQuant software (all from Molecular Dynamics, Sunnyvale, CA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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